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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No. 09/365,349
Applicant: Terry et al.
Filed: July 30, 1999
Docket No. B99-085
Title: *Heavy Metal Phytoremediation*

Customer No.: 23379
Confirmation No. 1676
Group Art Unit: 1638
Examiner: Ibrahim, Medina

CERTIFICATE OF TRANSMISSION
I hereby certify that this corr is being transmitted by facsimile to the Comm for Patent 571-273-8300 on November 29, 2005.

Signed _____
Richard Aron Osman

SUPPLEMENTAL BRIEF ON APPEAL

The Honorable Board of Appeals and Interferences
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Honorable Board:

We appeal from the Jun 16, 2005 final rejection of claims 1-24. This Supplemental Brief on Appeal is responsive to the Notice dated Nov 29, 2005 by adding the cited Arisi et al. (2000) reference to the evidence appendix.

REAL PARTY IN INTEREST

The real party in interest is The Regents of the University of California, the assignee of this application.

RELATED APPEALS AND INTERFERENCES

Appellants are unaware of any related appeals or interferences.

STATUS OF CLAIMS

Claims 1-24 are rejected and subject to this appeal.

STATUS OF AMENDMENTS

All Amendments are believed to be properly before the Board; an after-final amendment

filed Jul 25, 2005 was entered by Advisory Action dated Jul 28, 2005.

SUMMARY CLAIMED SUBJECT MATTER

The invention relates to a specific plant species *(Populus angustifolia, Nicotiana tabacum* or *Silene cucubalis)* that is genetically engineered to overexpress glutamylcysteine synthetase and thereby provides enhanced heavy metal accumulation as compared with a corresponding wild type plant. Specification, p.3, lines 19-20; p.6, lines 7-9.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

I. WHETHER THE EXAMINER HAS PROPERLY REJECTED CLAIMS 1 and 10 UNDER 35USC112, first paragraph (enablement).

ARGUMENT

I. THE EXAMINER HAS NOT PROPERLY REJECTED CLAIMS 1 and 10 UNDER 35USC112, first paragraph (enablement).

The previously appealed-from enablement rejection and the resultant Board Decision dated July 31, 2003 rely on Noctor et al.'s (1998 J Exp Bot 49, 623-647) reference to "preliminary experiments" wherein ECS-overexpressing poplars and non-transformed poplars accumulated Cd to a similar extent. The enablement rejection and Decision were expressly premised on an assumption: that "it would require an undue amount of experimentation to produce hyperaccumulating plants other than Brassica plants without further guidance from applicants as to why the construct produced a hyperaccumulating Brassica plant but failed to produce a hyperaccumulating poplar." Decision, p.14, lines 12-16.

As we explained in our prior Reply Brief, we do know why Noctor et al.'s early plants did not show hyper-accumulation:

> Noctor et al. did not have the benefit of our Specification, which teaches how to make the claimed hyper-accumulating plants, including hyper-accumulating poplars. Noctor et al reports that in unpublished "preliminary experiments" they failed to obtain hyper-accumulating poplars. We do not know how Noctor et al. did their experiments, so it is not possible for us to determine why they failed: we do not know in what form they provided the Cd, we

> do not know whether their poplars were subject to other variables that would have interfered with accumulation, we do not know how they made their transformants, we do not know whether their preliminary experiments were based on one or two anomalous plants, we do not know if their soil had other toxins or confounding microorganisms that may have independently depleted the supplemented Cd, etc. It is possible that the results of Noctor et al. are based on experimental error or contaminated materials. On the other hand, it is possible that they result merely from an insufficient sample size – had they generated sufficient data, they may well have obtained hyper-accumulators.
>
> Reply Brief p.2, lines 13-25; also quoted in Decision, para. bridging p.11 and 12.

Indeed, the same laboratory latter published their subsequent experiments (Arisi et al. 2000, Physiol Plant 109, 143-9, now of record). In these subsequent experiments, their ECS-overexpressing poplar did indeed provide higher cadmium accumulation than corresponding untransformed plants. (Arisi 2000; see abstract; para. bridging col. 1 and 2 of p.145; Fig.1). Of course, this subsequent report also had the benefit of the subject Applicant's intervening teachings, as reported in Zhu et al. Plant Physiol 119, 73-79, 1999 and Zhu et al., Plant Physiol 121, 1169-1177, **as cited, inter alia, on p.144, col.1, lines 34-37 of Arisi 2000.**

Arisi 2000 confirms that the disclosed methods produce a hyperaccumulating poplar as readily as they produce a hyperaccumulating Brassica plant. We attempted to provide this reference to the Board in a Request for Rehearing; however, the Board declined to consider evidence not previously made of record (Decision dated Sept 30, 2003, p.2, lines 5-8).

The enablement issue is whether the specification enables one of ordinary skill in the art to practice the invention as claimed without undue experimentation. Here, the product claims are drawn to a plant which is genetically engineered to overexpress glutamylcysteine synthetase and thereby provides enhanced heavy metal accumulation as compared with a corresponding wild type plant. The corresponding method claims require only two steps (a) identifying a medium as containing an excessive amount of a heavy metal; and (b) growing a subject plant in the medium, under conditions wherein the glutamylcysteine synthetase is overexpressed, whereby the plant provides enhanced accumulation of the heavy metal, whereby the heavy metal content of the medium is decreased.

The subject enablement rejection is limited to the scope of the recited plant. The claims

require a plant structurally limited to a plant genetically engineered to overexpress glutamylcysteine synthetase and functionally limited to one which does in fact overexpress the recited glutamylcysteine synthetase *and* thereby provides enhanced accumulation of the targeted heavy metal as compared with a corresponding wild type plant (see claim 1). The same claims, limited to Brassica plants, were allowed and issued in continuing application 09/933,549 (now US Patent No. 6,576,816). In an effort to expedite allowance, the pending claims have been limited to just three particularly preferred, alternative plant species: *Populus angustifolia*, *Nicotiana tabacum* and *Silene cucubalis*. Specification, p.6, lines 7-9. These are the same three plant species recited in Table 2, which was derived from an experimental research proposal to exemplify across a panel of defined alterative plant varieties and conditions the proof-of-principle experiments performed with Brassica plants and reported in the subsequent Experimental Protocols and Results section (p.8, line20 - p.13, line 17). Note that these claims are narrowly drafted to specific plant *species*, whereas the issued patent encompasses all plant species of the Brassica *genus*.

The invention is premised on Applicants' finding that the recited glutamylcysteine synthetase effects heavy metal accumulation, is causative of heavy metal accumulation and is rate-limiting of heavy metal accumulation. The disclosure establishes a predictable relationship between heavy metal exposure and overexpression of glutamylcysteine synthetase; namely, that such overexpression promotes enhanced accumulation of the metal. Accordingly, the specification aptly enables one of ordinary skill in the art to practice the method in any plant which is genetically engineered to overexpress glutamylcysteine synthetase and thereby provide enhanced accumulation of the heavy metal.

After our filing date, and citing publications describing our invention, Arisi 2000 reports successful heavy metal hyperaccumulation in a hybrid poplar overexpressing overexpress glutamylcysteine synthetase. Though not the particular poplar species of our claims, this study appears the undermine the rationale of the rejection, particularly as applied to claims 3 and 24 which are limited to a poplar species. Please note that the poplars of Arisi 2000 were newly generated in that 2000 study by micropropogation in vitro (Arisi 2000, p.144, para. bridging col.1 and col.2). The poplars of Arisi 1997 (Planta 203, 362-372) are not the same plants studied in Arisi 2000. Hence, the Action misstates the facts when it alleges that "the transformed plants of Arisi et al (1997) and Arisi (2000) are identical." Action p.6, last line. While the 2000 plants

derived from previously produced clones, they are not the same plants, and were grown under different standards than the poplars of Arisi 1997.

We are also unable to concur with the Action's proposal at p.7, lines 3 - 19 that reduced heavy metal tolerance is inconsistent with enhanced accumulation. In fact, these two properties often correlate, as discussed in the manuscript entitled "Expression of a High-Affinity Sulfate Transporter in *Brassica juncea* Affects Metal Tolerance and Accumulation" (Lindblom, Abdel-Ghany, Hanson, Hwang, Terry, and Pilon-Smits, 2005, submitted for publication, of record). In any event, our claims are indifferent to whether the plants display increased or decreased heavy metal tolerance.

For good measure, we have of record an expert declaration from a University of California Professor averring to the foregoing. The Declarant/Professor is knowledgeable of the dispositive factual determination of what one skilled in this art would and would not consider undue experimentation. This Declaration is authoritative evidence of a documented expert in the field of what the application enables one skilled in this art.

Appellants respectfully request reversal of the pending Final Action by the Board of Appeals.

The Appeal Brief Fee is provided in the attached PTO-2038. We petition for and authorize charging our Deposit Account No.19-0750 all necessary extensions of time. The Commissioner is authorized to charge any fees or credit any overcharges relating to this communication to our Dep. Acct. No.19-0750 (order B99-085).

Respectfully submitted,
SCIENCE & TECHNOLOGY LAW GROUP

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EVIDENCE APPENDIX

The following Declaration of Professor Feldman under 37CFR1.132 was entered in the record with our Response filed Sep 20, 2004, and acknowledged by the Examiner in her Dec 22, 2004 Action at p.13, line 3.

The following Arisi et al. (2000, Physiol Plant 109, 143-9) reference was entered in the record with our RCE filed Oct 17, 2005, and acknowledged by the Examiner in her Apr 20, 2004 Action.

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Serial No. 09/365,349	Examiner: Ibrahim, M.
Filed: July 30, 1999	
For: *Heavy Metal Phytoremediation*	Attorney Docket No. B99-085

DECLARATION UNDER RULE 132

I, Lewis Feldman, declare and state as follows:

1. I am a Professor in the Department of Plant and Microbial Biology at the University of California, Berkeley. The Regents of the University of California is the assignee of the subject patent application. I am knowledgeable and experienced in the field of genetic engineering in plants. I have read and am familiar with the contents of the above application.

2. The product claims of this application are drawn to a plant which is genetically engineered to overexpress glutamylcysteine synthetase and thereby provides enhanced heavy metal accumulation as compared with a corresponding wild type plant. The corresponding method claims require only two steps (a) identifying a medium as containing an excessive amount of a heavy metal; and (b) growing a subject plant in the medium, under conditions wherein the glutamylcysteine synthetase is overexpressed, whereby the plant provides enhanced accumulation of the heavy metal, whereby the heavy metal content of the medium is decreased.

The specification teaches that "a wide variety of plants may be used, as urged by the particular trace element, medium, site geology, topology, weather, etc. Additional factors for selection include large biomass production, relatively high trace element accumulation capacity, and ease of genetic engineerability", citing Zhu et al., 1999, Plant Physiol 119:73-79. Specification, p.4, lines 6-9. The claims are structurally limited to a plant genetically engineered to overexpress glutamylcysteine synthetase and functionally limited to one which does in fact overexpress the recited glutamylcysteine synthetase *and* thereby provides enhanced accumulation of the targeted heavy metal as compared with a corresponding wild type plant (see

claim 1). "Suitable plants are readily screened for requisite engineerability and expression from examplars of candidate plant varieties by those skilled in the art of plant genetic engineering, as exemplified below." Specification, p.4, lines 9-11. The specification offers a large number of suitable, commercially available varieties of exemplary plant source materials (p.4, line 11 - p.6, line 9). Furthermore, the specification describes diverse exemplary plant species demonstrating enhanced elemental assimilation in wild-type plants and the corresponding plant overexpressing a variety of recombinant glutamylcysteine synthetase genes (p.7, line 26 - p.8, line 18); exemplified plants include Brassica juncea, Populus angustifolia, Nicotiana tabacum and Silene cucubalis. The suitability of any given plant is readily ascertained by simple substitution into the same method.

The invention is premised on Applicants' finding that the recited glutamylcysteine synthetase effects heavy metal accumulation, is causative of heavy metal accumulation and is rate-limiting of heavy metal accumulation. The disclosure establishes a predictable relationship between heavy metal exposure and overexpression of glutamylcysteine synthetase; namely, that such overexpression promotes enhanced accumulation of the metal. This relationship is shown to hold across numerous and diverse exemplary plant species (supra). Accordingly, as an expert in the field, it is my opinion that the specification aptly enables one of ordinary skill in the art to practice the method in any plant which is genetically engineered to overexpress glutamylcysteine synthetase and thereby provide enhanced accumulation of the heavy metal.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application and any patent issuing therefrom.

Date: 9/20/04

Prof. Lewis Feldman

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Responses to cadmium in leaves of transformed poplars overexpressing γ-glutamylcysteine synthetase

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Poplars overexpressing a bacterial γ-glutamylcysteine synthetase (γ-ECS) in the cytosol (lines ggs11 and ggs28) had a 30-fold increase in foliar γ-ECS activity relative to untransformed controls. Foliar γ-glutamylcysteine (γ-EC) was increased by 10-fold while foliar glutathione accumulation increased by up to 3.5-fold in the leaves of the transformants. Untransformed and transformed poplars were grown with different soil concentrations of cadmium (0–1100 μg g^{-1} soil) for 2 weeks. Cadmium accumulated in the leaves of both transformed and untransformed poplars and growth was inhibited. Growth inhibition and foliar cadmium accumulation were greatest at the highest soil cadmium concentrations in all lines. Exposure to cadmium enhanced the foliar cysteine, γ-EC and glutathione pools in all lines but less glutathione was present in the leaves of the untransformed controls than the transformants under all growth conditions. Cadmium-induced changes in the activities of malic enzyme, isocitrate dehydrogenase and guaiacol peroxidase were less pronounced in the leaves of the transformed poplars overexpressing γ-ECS than in the untransformed controls. Glutamate dehydrogenase and glutathione reductase activities were unchanged by exposure to cadmium. We conclude that overexpression of γ-ECS activity and foliar glutathione accumulation in transformed poplar allows greater tissue cadmium accumulation but has only a marginal effect on cadmium tolerance in poplar.

Introduction

Cadmium is a pollutant that accumulates in soil as a result of industrial processes or intensive use of fertilisers in agriculture. Its phytotoxicity is related to its reactivity with O-, N- and S-containing ligands (Van Assche and Clijsters 1990a). Cd inhibits photosynthesis (Clijsters and Van Assche 1985) but stimulates respiration. The activities of the tricarboxylic acid cycle and of other pathways of carbohydrate utilisation are induced by Cd accumulation in leaves. This is related to increased demand for ATP production by oxidative phosphorylation to compensate for deficits in photophosphorylation (Ernst 1980). In particular, the activities of isocitrate dehydrogenase (ICDH), malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), malic enzyme (ME), glucose 6-phosphate dehydrogenase and peroxidases (POD) are increased following Cd exposure (Van Assche and Clijsters 1990a).

Cd induces the synthesis of cysteine-rich peptides with the general structure $(\gamma\text{-EC})_nG$, called phytochelatins (Rauser 1995), and of other thiol peptides $(\gamma\text{-EC})_n$ and $(\gamma\text{-EC})_nE$ (Meuwly et al. 1995). Phytochelatins form complexes with Cd in the cytosol and are important in subsequent Cd sequestration in the vacuoles (Ortiz et al. 1995). They participate in the maintenance of cellular metal homeostasis (Zenk 1996) and are involved in limiting the transport of heavy metal ions from roots to shoots (Galli et al. 1996). Phytochelatins are synthesised by γ-EC dipeptidyl transpeptidase from the precursor reduced glutathione (GSH; Grill et al. 1989). GSH is synthesised by two sequential reactions,

Abbreviations – γ-ECS, γ-glutamyl cysteine synthetase; GDH, glutamate dehydrogenase; GR, glutathione reductase; GS, glutathione synthetase; GSH, reduced glutathione; GSSG, oxidised glutathione; ICDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; POD, peroxidase.

catalysed by γ-glutamyl cysteine synthetase (γ-ECS) and glutathione synthetase (GS) in the chloroplasts and cytosol of plant cells (Noctor et al. 1998a,b).

In addition to phytochelatins, metallothioneins are cysteine-rich peptides that play a major role in metal detoxification (Zhou and Goldsbrough 1994). Overexpression of metallothionein genes in transformed plants has been used to study Cd tolerance. Expression of a mammalian metallothionein cDNA in transformed tobacco plants resulted in improved Cd resistance (Pan et al. 1994) and in reduced Cd accumulation in leaves (Maiti et al. 1991, Elmayan and Tepfer 1994). Several studies have shown that tolerance to Cd is also related to GSH accumulation in leaves and an increased capacity of GSH synthesis. A Cd-sensitive mutant of *Arabidopsis thaliana*, deficient in the ability to sequester Cd, was shown to have very low glutathione contents (Howden et al. 1995). GSH was decreased in the roots of pea plants cultivated with Cd (Klapheck et al. 1995). In maize, Cd exposure led to decreased GSH and increased γ-EC in both roots and shoots (Rauser et al. 1991, Rüegsegger and Brunold 1992) and to increases in the maximal extractable γ-ECS activity of roots (Rüegsegger and Brunold 1992). GSH depletion and γ-EC accumulation were also observed in parsley and tobacco cell cultures following Cd treatment. γ-ECS and GS activities were found to increase in tobacco cell cultures treated with Cd (Schneider and Bergmann 1995). Cultured tomato cells, selected for increased Cd tolerance, had increased γ-ECS activity (Chen and Goldsbrough 1994). Cd exposure of *Arabidopsis* plants activated transcription of the genes for glutathione synthesis (Xiang and Oliver 1998). Furthermore, buthionine sulphoximine, an inhibitor of γ-ECS, inhibited phytochelatin accumulation (Grill et al. 1987) and enhanced the Cd-dependent growth inhibition in plants (Gussarsson et al. 1996). Transformed Indian mustard plants overexpressing either GS (Zhu et al. 1999a) or γ-ECS (Zhu et al. 1999b) accumulated more Cd than untransformed plants and showed enhanced tolerance.

We have produced transformed poplars overexpressing the *Escherichia coli* γ-ECS in the cytosol (Noctor et al. 1996, Arisi et al. 1997, Noctor et al. 1998a). These transformed plants have constitutively increased foliar glutathione contents compared with untransformed poplars, whereas GS overexpression did not modify foliar GSH contents of poplars (Strohm et al. 1995, Noctor et al. 1998b). γ-ECS is the rate-limiting enzyme for glutathione biosynthesis in poplars. To investigate the role of enhanced γ-ECS activity and glutathione in protection against Cd-induced inhibition of metabolism, transformed and untransformed poplars were grown for 2 weeks at different Cd concentrations. This is the first report of Cd-induced changes in foliar thiol contents, the activities of POD and of enzymes related to carbon utilisation in transformed plants with increased γ-ECS activity.

Materials and methods

Plant material

Untransformed (WT) and transformed hybrid poplars (*Populus tremula* × *P. alba*; Institut National de la Recherche Agronomique No. 717-1-B4, Versailles, France), overexpressing γ-glutamylcysteine synthetase in the cytosol, were micropropagated in vitro. The two lines (ggs11 and ggs28) of transformed poplar used in the present work had high extractable foliar γ-ECS activities relative to untransformed poplar (Arisi et al. 1997). The plants were transferred to pots containing artificial soil (70% quartz sand, 20% kaolin, 10% ground peat, 0.5% $CaCO_3$) and introduced in the greenhouse, where they were regularly watered with nutrient solution. After 6 weeks in the greenhouse, plants were put into bigger pots containing 475 g of artificial soil per pot and then transferred to a controlled environment chamber with a 16-h photoperiod (300 μmol m^{-2} s^{-1} irradiance), 25/20°C day/night and 75% relative humidity. Plants were fed with nutrient solution (1 m*M* $NH_4H_2PO_4$, 5.6 m*M* KNO_3, 4.0 m*M* $Ca(NO_3)_2$, 2.0 m*M* $MgSO_4$, 46.3 μ*M* H_3BO_3, 9.1 μ*M* $MnCl_2$, 64.7 μ*M* $FeSO_4$, 0.3 μ*M* $CuSO_4$, 0.76 μ*M* $ZnSO_4$, 0.52 μ*M* H_2MoO_4) every 3 days and watered several times a day to maintain the water status at between 50 and 70% of the retention capacity. For analysis of foliar metabolism in all the following experiments, only the youngest mature leaves were used.

Cadmium treatment

One week after the transfer to the controlled environmental chamber, the height of the plants was measured and the 5th leaf from the top was tagged. Then, $Cd(NO_3)_2$ solution was added to the soil in order to obtain the following Cd concentrations in the soil: 0, 100, 300, 500, 700, 900, 1100 μg g^{-1} dry weight. After 2 weeks of Cd treatment, the height of the plants was measured and the number of new leaves that appeared at the top was determined. Three plants at each Cd concentration from each poplar line (WT, ggs11 and ggs28) were harvested and washed in distilled water. Each plant was divided into roots, bottom leaves (below and including the tagged 5th leaf), medium leaves (4th, 3rd and 2nd, which had enlarged during the treatment), upper leaves (3 following leaves which appeared and enlarged during the treatment), top leaves (small leaves that had appeared above the upper leaves) and remaining shoot and petioles. For each treatment, roots, medium leaves and upper leaves were pooled and then cut with ceramic scissors. The resulting small pieces were sampled (0.5 g fresh weight) and the samples were immediately frozen in liquid N_2 and stored at −80°C for thiol and enzyme measurements. The remaining leaf pieces and other lower parts were weighed and oven-dried at 80°C to constant weight for determining dry weight and cadmium content.

Metal analysis

Oven-dried plant samples were heated for several hours at 470°C in an oven. The resulting ashes were solubilised in 5% HNO_3 (50 ml final volume per 0.5 g dry weight). Cd concentration in digested solutions was determined by inductively coupled plasma emission (Varian Liberty 200, Victoria, Australia). Quality control for plant analysis was performed by analysing blanks and certified reference material (Ryegrass BCR 281, Community Bureau of Reference, Commission of the European Communities) using the same method in triplicate.

Determination of thiols

Cysteine, γ-glutamylcysteine (γ-EC) and GSH were extracted in acid from samples frozen at −80°C. Samples of root and shoot tissues (0.5 g fresh weight) were ground in a mortar with liquid nitrogen, then, 100 mg insoluble polyvinylpyrrolidone and 5 ml 0.1 *M* HCl, 1 m*M* EDTA were added. Thiol contents were determined fluorimetrically as monobromobimane derivatives following separation by reverse-phase HPLC, as described in Arisi et al. (1997). Oxidised glutathione (GSSG) was assayed as described in Foyer et al. (1995), after the method of Griffith (1980).

Enzyme assays

Frozen material (0.5 g fresh weight) was homogenised with an ice cold mortar and pestle in 2.5 ml of 0.1 *M* Tris-HCl buffer (pH 7.8), containing 1 m*M* dithiothreitol and 1 m*M* EDTA. The homogenate was squeezed through a nylon mesh and centrifuged at 12000 *g* at 4°C for 10 min. The supernatant was collected and the activities of ME, ICDH and GDH were measured spectrophotometrically with a Varian Cary 1E spectrophotometer as described by Van Assche et al. (1988). POD was assayed by the method of Van Assche and Clijsters (1990b) and glutathione reductase (GR) was assayed as described by Foyer and Halliwell (1976).

Anionic (iso)peroxidases were separated by polyacrylamide gel electrophoresis on 7.5–20% gradient gels. POD activity was detected by incubation with 0.04% benzidine and 0.006% (v/v) H_2O_2 for 1.5 h at 37°C (Van Assche and Clijsters 1990b) and bands of POD activity quantitated by densitometry at 632 nm.

Determination of relative growth and statistical analysis

The height of each shoot was measured from the plant/soil interface to the uppermost leaf. Relative growth (RG) was determined as the final plant height minus the initial height divided by the initial height and expressed as a percentage. All lines were fitted using software from Biosoft, Cambridge, UK applying the least-squares method.

Results

Leaf Cd concentration and plant growth

Poplars overexpressing γ-ECS in the cytosol (ggs11 and ggs28) and untransformed (WT) poplars were grown for 2 weeks in the presence of Cd (0–1100 μg g^{-1} dry weight soil). In all plant types, foliar Cd accumulation increased in relation to the Cd content of the soil as shown in Fig. 1. Foliar Cd contents increased sharply relative to soil Cd content at low soil Cd concentrations (0–200 μg g^{-1} dry weight). For soil Cd contents between 200 and 900 μg g^{-1} dry weight, foliar Cd contents were only slightly increased. A large increase in foliar Cd occurred in plants grown at soil Cd concentrations between 900 and 1100 μg g^{-1} dry weight. At high soil Cd concentrations (1100 μg g^{-1} dry weight), the transformed poplars tended to show higher foliar cadmium accumulation than the untransformed controls. Similar results were obtained for upper and lower leaves on the trees.

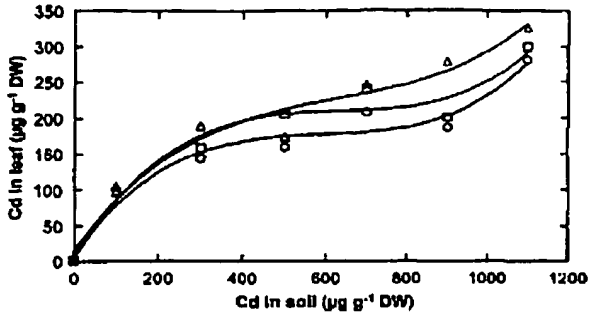

Fig. 1. Foliar Cd accumulation in untransformed (○) and transformed (△, ggs11; □, ggs28) poplars after 2 weeks growth in the presence of different soil Cd concentrations.

Cd accumulation caused decreased growth in all plant types (Fig. 2A). Cd-dependent growth inhibition was greatest in plants grown with soil Cd concentrations above 500 μg g^{-1} dry weight (corresponding to 150–200 μg g^{-1} dry weight in the leaves) and was most marked in transformed poplars. The effect of Cd on relative growth rate was similar in all leaves (Fig. 2B) indicating that Cd-induced effects on total new leaf biomass were comparable in all plants.

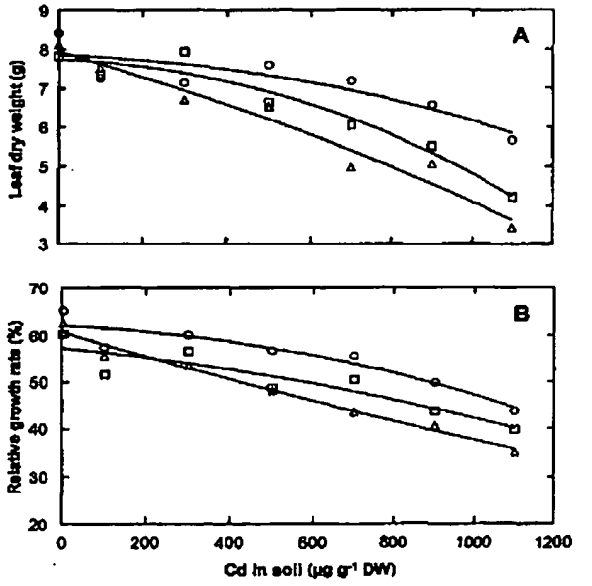

Fig. 2. Foliar dry weight (A) and shoot relative growth rates (B) of untransformed (○) and transformed (△, ggs11; □, ggs28) poplars after 2 weeks growth in the presence of different soil Cd concentrations.

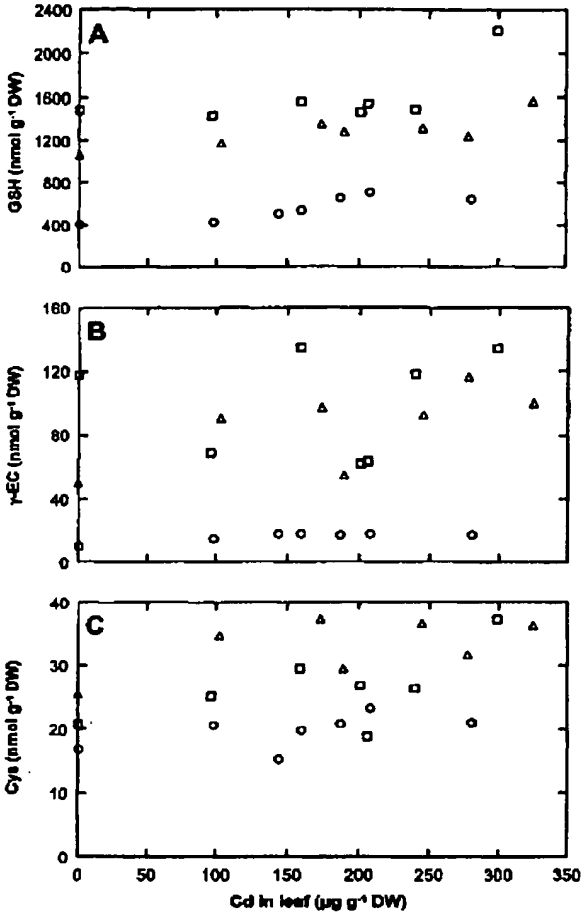

Fig. 3. Foliar thiol accumulation in untransformed (O) and transformed (Δ, ggs11; □, ggs28) poplars relative to foliar leaf Cd contents. Plants were harvested after 2 weeks growth in the absence and presence of different Cd concentrations (A) foliar glutathione (GSH) (B) γ-glutamyl cysteine (γ-EC) and (C) cysteine (Cys). The results presented are the mean values of at least two determinations performed on pooled leaf samples from 3 individual plants.

Effect of Cd on thiol contents

Leaves from transformed poplars overexpressing γ-ECS (lines ggs11 and ggs28) had 2.5- and 3.5-fold more GSH in their leaves, respectively, than those of untransformed (WT) poplars in the absence of cadmium (Fig. 3; Arisi et al. 1997). The roots of these transformed plants also had approximately 3 times more GSH (A. C. M. Arisi 1997. Thesis, Université de Paris-Sud UFR Scientifique d'Orsay, France). After 2 weeks growth with Cd, the GSH content of the roots was similar in all plants (data not shown) and not changed by the presence of Cd in the soil. The GSH contents of the leaves were increased, however, in all plant types. The increase was proportional to the Cd content of the leaves when the foliar Cd content was over 100 μg Cd g^{-1} dry weight leaf. At higher levels of foliar Cd accumulation, foliar GSH content in untransformed poplars increased 400–700 nmol g^{-1} dry weight of untransformed plants, but this value was much less than the GSH content of the leaves of the transformants. GSH exceeded 1000 nmol g^{-1} dry weight in lines ggs11 and ggs28 (Fig. 3). Even in the transformed poplars, GSH increased as a result of Cd exposure, in ggs11 GSH increased from 1040 to 1540 nmol g^{-1} dry weight in the presence of Cd. Foliar GSH increased only at the highest foliar Cd concentrations (300 μg g^{-1} dry weight) in line ggs28 to a maximum of 2160 nmol GSH g^{-1} dry weight. It is important to note that ggs28 plants showed the highest foliar GSH contents prior to Cd treatment and was only exceeded by ggs11 when foliar Cd reached 320 μg g^{-1} dry weight. The GSH/GSSG ratio was 9/1 in leaves for all plants under all growth conditions (Arisi et al. 1997) and was not changed by Cd treatment (data not shown).

Foliar γ-EC was up to 10-fold higher in lines ggs11 and ggs28 relative to untransformed controls (Fig. 3). Cd exposure caused a 2-fold increase of γ-EC in the untransformed poplars. However, these values were still much less than those obtained with lines ggs11 and ggs28 without the Cd treatment (50 and 120 nmol g^{-1} dry weight, respectively; Fig. 3). Foliar γ-EC contents were enhanced following Cd treatments. Similar results were obtained for upper and lower leaves (data not shown).

Transformed poplar leaves overexpressing γ-ECS generally contained more cysteine than those from untransformed plants (Arisi et al. 1997, Noctor et al. 1998a). The cysteine pool in leaves prior to Cd exposure amounted to 17, 26 and 20 nmol g^{-1} dry weight in untransformed poplars, lines ggs11 and ggs28, respectively (Fig. 3). Foliar cysteine increased with increasing foliar Cd contents in all plants (Fig. 3), especially above 150 μg Cd g^{-1} dry weight.

Effect of Cd on enzyme activities

In the upper leaves, an increase in the activities of the NAD(P)H-producing enzymes ME and ICDH was observed as Cd accumulated in the leaves (Fig. 4). The activities of these enzymes in the absence of Cd were similar in untransformed and transformed lines (Fig. 4). ME activity was increased to values 2.5-fold higher in ggs11 and ggs28 and 4-fold higher in the untransformed poplars than leaves of plants grown in the absence of Cd. Cd-induced increases in ICDH activity were 2- and 3-fold, respectively, in transformed lines and in untransformed controls.

Foliar POD activity (Fig. 4) was lower in the transformed lines (105 mU mg^{-1} protein) than the untransformed plants (140 mU mg^{-1} protein) in the absence of Cd. An increase in POD activity was observed in untransformed poplars and ggs28 at foliar Cd concentrations above 150 μg g^{-1} dry weight, and in ggs11 at foliar Cd concentration of 100 μg g^{-1} dry weight. Cd-induced changes in total foliar POD activity were not reflected by a change in the pattern of isoenzyme activity bands revealed by activity staining on gels following electrophoresis, even at the highest Cd con-

centrations (data not shown). The intensity of all POD activity bands was increased uniformly (data not shown).

The activities of GDH and GR were not modified by Cd exposure (data not shown).

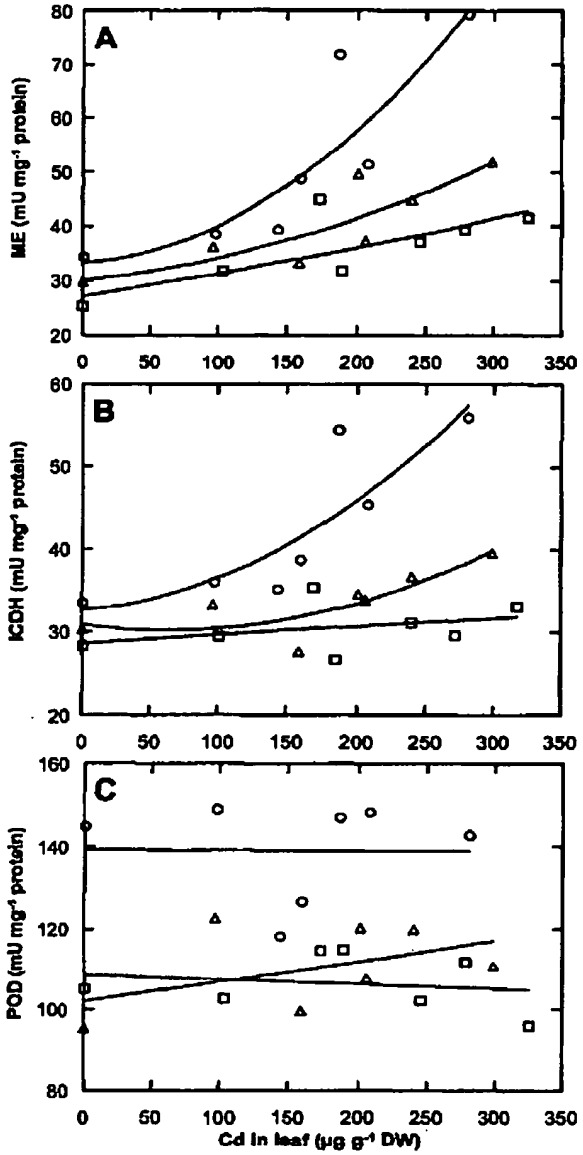

Fig. 4. Foliar activities of (A) malic enzyme (ME) (B) isocitrate dehydrogenase (ICDH) and (C) guaiacol peroxidase (POD) from untransformed (○) and transformed (△, ggs11; □, ggs28) poplars relative to foliar leaf Cd contents. Plants were harvested after 2 weeks growth in the presence of increasing Cd concentrations. The results presented are the mean values of at least two determinations performed on pooled leaf samples from 3 individual plants.

Discussion

The effects of cadmium on growth and metabolism were studied in untransformed poplars and in transformed poplars overexpressing γ-ECS with constitutively enhanced tissue GSH contents (Noctor et al. 1998a). Leaves of the transformed lines had a slightly higher Cd content than those of untransformed controls, particulary at high soil Cd concentrations (Fig. 1). The accumulation of Cd was associated with a proportionally greater Cd-induced inhibition of growth in the transformants (Fig. 2). Analysis of morphological markers, such as biomass and relative growth rate, suggests that the untransformed controls tolerated more Cd than the transformed lines. Transformed Indian mustard overexpressing either GS or γ-ECS accumulated more Cd than untransformed plants and were more tolerant to Cd (Zhu et al. 1999a,b). While the ME, POD and ICDH data obtained in the present work suggest that metabolism in the γ-ECS transformants is less perturbed by Cd than in the untransformed controls, these results do not provide conclusive proof that Cd tolerance is increased by γ-ECS overexpression.

Exposure to Cd led to increases in foliar γ-EC and GSH accumulation in all poplar lines, irrespective of the GSH content of the leaves in the absence of Cd. An increase in foliar GSH was observed in maize leaves at leaf Cd concentrations above 3.4 µg g^{-1} dry weight (Lagriffoul et al. 1997); but, in poplars, the increase in GSH occurred only at much higher Cd values. The factors responsible for the Cd-induced increases in thiols merit attention, since it is clear that GSH synthesis is under multifactorial control (Noctor et al. 1998a). Treatment of maize roots and leaves with Cd led to increased γ-EC contents and enhanced rates of GSH synthesis (Rüegsegger and Brunold 1992). Similar observations were reported on exposure of cultured cells to Cd (Schneider and Bergmann 1995). In these previous studies, the increases in thiols were accompanied by increases in the extractable activities of γ-ECS (Rüegsegger and Brunold 1992) and GS (Schneider and Bergmann 1995). Increases in γ-ECS are likely to be particularly important, since overexpression studies strongly suggest that the major control over GSH accumulation resides with this enzyme (Noctor et al. 1996, Arisi et al. 1997, Noctor et al. 1998a,b). In both animal and plant cells, Cd treatment induced increases in γ-ECS gene transcription (Hatcher et al. 1995, Xiang and Oliver 1998). Thus, up-regulation of γ-ECS may well have contributed to the observed increases in GSH in untransformed poplars. However, the transformants used in the present work have extractable γ-ECS activities which, even in the absence of Cd, are higher than those induced by Cd in maize plants and tobacco cells (Rüegsegger and Brunold 1992, Schneider and Bergmann 1995). It is unclear, therefore, whether the slight increases in thiols in the ggs transformants can be explained by up-regulation of γ-ECS. Overexpression of γ-ECS causes the control of GSH synthesis to shift to the second enzyme, GS (Noctor et al. 1998a). It is possible that induction of this enzyme activity, as observed in Cd-exposed pea roots (Rüegsegger et al. 1990) and tobacco cells (Schneider and Bergmann 1995), may have contributed to the Cd-induced GSH accumulation in the

poplar transformants. In support of this view, Zhu et al. (1999a) have demonstrated that overexpression of GS enhances cadmium accumulation in *Brassica juncea*. Thus, γ-ECS would appear to be the rate-limiting step in glutathione biosynthesis in the absence of Cd, while exposure to Cd shifts the equilibrium so that GS also limits GSH production.

Whatever the factors responsible for the increases in thiols, the present data confirm that induction of GSH biosynthesis seems to be an early inducible mechanism of protection against toxic heavy metal accumulation. This protection would presumably be manifested through an effect of increased foliar GSH on the synthesis of phytochelatins. Phytochelatins are known to inactivate Cd in the cytoplasm and facilitate the transport of Cd into the vacuole. Although direct measurements of foliar phytochelatin contents were beyond the scope of the present work, the high GSH content of leaves and roots in the transformed plants should confer a greater capacity for phytochelatin synthesis, enabling formation of more thiolate bonds with Cd. In *Brassica juncea* exposed to Cd, the expression of γ-ECS was closely correlated with phytochelatin synthesis (Haag-Kerwer et al. 1999). The increased accumulation of Cd in the leaves of the transformants suggests that there is some enhancement of phytochelatin synthesis in the leaves of these plants relative to controls.

The activities of enzymes associated with energy metabolism and regeneration of reducing power (NADPH and NADH) were increased by Cd treatment. ME and ICDH activities were similar in untransformed and transformed plants in the absence of Cd, suggesting that the transformation procedures have not modified the general energy metabolism of the poplar cells. The Cd-induced increases in ME and ICDH activities were higher in the leaves of the untransformed poplars at the highest foliar Cd concentrations. Increases in enzyme activity were observed at foliar Cd concentrations above 150 μg g^{-1} dry weight in all plant types. This value is high compared with values of Cd that induce increases in enzyme activity in bean leaves (4.6 and 5.5 μg g^{-1} dry weight, respectively, for ME and ICDH; Van Assche et al. 1988). Conversely, maize leaves showed decreased ICDH activity at Cd concentrations above 22 μg g^{-1} dry weight and no significant Cd-induced changes in ME activity were observed (Lagriffoul et al. 1997).

An increase in POD activity above foliar Cd concentrations of 150 μg g^{-1} dry weight was observed. As for the activities of other enzymes, the increase in POD activity was highest in the untransformed poplars. POD activity was found to be induced in bean and maize leaves at lower Cd concentrations than in poplar: 5.5 μg g^{-1} dry weight in bean (Van Assche et al. 1988) and 3–5 μg g^{-1} dry weight in maize (Lagriffoul et al. 1998). In poplars, as in maize, no induction of new POD isoenzymes was found. In contrast, two minor novel bands of POD activity were observed in bean leaves following Cd treatment (Van Assche and Clijsters 1990a). It is interesting to note that no changes in GR activity were observed in Cd-treated plants, in contrast to previous observations in maize (Lagriffoul et al. 1997). GR activity in poplars may be sufficiently high to maintain the balance between oxidised and GSH in the presence of Cd, requiring no further induction of GR activity. Similarly, no Cd-induced changes in GDH activity were observed. This latter observation is similar to previous studies in maize (Lagriffoul et al. 1997), but in contrast to work in bean leaves, where an induction of GDH activity was observed following Cd treatment (Van Assche et al. 1988). Overexpression of either γ-ECS or GS increased Cd accumulation and tolerance in Indian Mustard. In transformed poplars, enhanced tissue γ-EC and GSH do not confer increased tolerance to Cd in terms of amelioration of growth. However, overexpression γ-ECS markedly decreased Cd-dependent activation of enzymes such as ME, POD and ICDH in the transformed poplars. Metabolism was, therefore, less perturbed by Cd in leaves overexpressing γ-ECS than in untransformed controls, probably because Cd was complexed in a non-toxic form in the transformants.

The leaves of the transformed poplars contained somewhat more Cd than the untransformed controls, suggesting potential importance for phytoremediation. The results presented here largely agree with those of Zhu et al. (1999a,b). We conclude that simultaneous overexpression of γ-ECS and GS, to enhance GSH production and accumulation even further (Noctor and Foyer 1998), is a promising strategy for improved heavy metal phytoremediation capacity.

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RELATED PROCEEDINGS APPENDIX

No related proceedings are known to exist.

CLAIMS APPENDIX

1. A plant which is a *Populus angustifolia*, *Nicotiana tabacum* or *Silene cucubalis* and is genetically engineered to overexpress glutamylcysteine synthetase and thereby provides enhanced heavy metal accumulation as compared with a corresponding wild type plant.

2. A plant according to claim 1 comprising a nucleic acid encoding the glutamylcysteine synthetase operably linked to a heterologous promoter.

3. A plant according to claim 1 which is a *Populus angustifolia*.

4. A plant according to claim 1 which is a *Nicotiana tabacum*.

5. A plant according to claim 1 wherein the heavy metal is selected from the group consisting of chromium, molybdenum and tungsten.

6. A plant according to claim 1 wherein the heavy metal is selected from the group consisting of cadmium and mercury.

7. A plant according to claim 1 wherein the heavy metal is uranium.

8. A plant according to claim 1, wherein the enhanced accumulation is at least 50% greater than an otherwise comparable untransformed plant.

9. A plant according to claim 1, wherein the plant comprises a nucleic acid encoding the glutamylcysteine synthetase operably linked to a heterologous promoter, the heavy metal is selected from the group consisting of chromium, molybdenum and tungsten and the enhanced accumulation is at least 50% greater than an otherwise comparable untransformed plant.

10. A plant according to claim 1, wherein the plant comprises a nucleic acid encoding the glutamylcysteine synthetase operably linked to a heterologous promoter, the heavy metal is selected from the group consisting of cadmium and mercury and the enhanced accumulation is at least 50% greater than an otherwise comparable untransformed plant.

11. A plant according to claim 1, wherein the plant comprises a nucleic acid encoding the glutamylcysteine synthetase operably linked to a heterologous promoter, the heavy metal is selected from the group consisting of tellurium and polonium and the enhanced accumulation is at least 50% greater than an otherwise comparable untransformed plant.

12. A plant according to claim 1, wherein the plant comprises a nucleic acid encoding the glutamylcysteine synthetase operably linked to a heterologous promoter, the heavy metal is uranium and the enhanced accumulation is at least 50% greater than an otherwise comparable untransformed plant.

13. A method for decreasing heavy metal content of a medium, comprising the steps of: (a) identifying a medium as containing an excessive amount of a heavy metal; and (b) growing a plant according to claim 1 in the medium, under conditions wherein the glutamylcysteine synthetase is overexpressed, whereby the plant provides enhanced accumulation of the heavy metal, whereby the heavy metal content of the medium is decreased.

14. A method for decreasing heavy metal content of a medium, comprising the steps of: (a) identifying a medium as containing an excessive amount of a heavy metal; and (b) growing a plant according to claim 7 in the medium, under conditions wherein the glutamylcysteine synthetase is overexpressed, whereby the plant provides enhanced accumulation of the heavy metal, whereby the heavy metal content of the medium is decreased.

15. A method for decreasing heavy metal content of a medium, comprising the steps of: (a) identifying a medium as containing an excessive amount of a heavy metal; and (b) growing a plant according to claim 8 in the medium, under conditions wherein the glutamylcysteine synthetase is overexpressed, whereby the plant provides enhanced accumulation of the heavy metal, whereby the heavy metal content of the medium is decreased.

16. A method for decreasing heavy metal content of a medium, comprising the steps of: (a) identifying a medium as containing an excessive amount of a heavy metal; and (b) growing a plant according to claim 9 in the medium, under conditions wherein the glutamylcysteine synthetase is overexpressed, whereby the plant provides enhanced accumulation of the heavy metal, whereby the heavy metal content of the medium is decreased.

17. A method for decreasing heavy metal content of a medium, comprising the steps of: (a) identifying a medium as containing an excessive amount of a heavy metal; and (b) growing a plant according to claim 10 in the medium, under conditions wherein the glutamylcysteine synthetase is overexpressed, whereby the plant provides enhanced accumulation of the heavy metal, whereby the heavy metal content of the medium is decreased.

18. A method for decreasing heavy metal content of a medium, comprising the steps of: (a) identifying a medium as containing an excessive amount of a heavy metal; and (b) growing a plant according to claim 11 in the medium, under conditions wherein the glutamylcysteine synthetase is overexpressed, whereby the plant provides enhanced accumulation of the heavy metal, whereby the heavy metal content of the medium is decreased.

19. A method according to claim 13, wherein the medium is soil.

20. A plant according to claim 1 wherein the plant grows not significantly differently than a corresponding wild type plant under non-heavy metal conditions.

21. A plant according to claim 4 wherein the plant grows not significantly differently than a corresponding wild type plant under non-heavy metal conditions.

22. A method according to claim 13 wherein the plant grows not significantly differently than a corresponding wild type plant under non-heavy metal conditions.

23. A plant which is a commercially available variety of *Silene cucubalis* and is genetically engineered to overexpress glutamylcysteine synthetase and thereby provides enhanced heavy metal accumulation as compared with a corresponding wild type plant.

24. plant which is a commercially available variety of *Populus angustifolia* and is genetically engineered to overexpress glutamylcysteine synthetase and thereby provides enhanced heavy metal accumulation as compared with a corresponding wild type plant.